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14. ABSTRACT

The development of drug resistance represents a formidable barrier to the successful treatment of breast cancer. Although some cancers such as melanoma can be intrinsically resistant, many cancers acquire resistance through selection pressure in the face of adversity, e.g., chemotherapy. One of the most consistent features of drug resistance is overexpression of P-glycoprotein (P-gp). This protein functions as a pump to reduce the intracellular concentration of anticancer drugs. Clinical use of P-gp antagonists to inhibit drug efflux has been disappointing. Here we propose silencing the multidrug resistance (MDR1) phenotype by retarding glycolipid metabolism via inhibition of glucosylceramide synthase (GCS), a lipogenic enzyme associated with MDR1. We will determine whether inhibitors of GCS affect MDR1/P-gp expression and chemotherapy sensitivity in drug-resistant breast cancer cells and determine whether GCS inhibitors forestall acquired resistance to chemotherapy in wild-type breast cancer cells. This is the first study to attack drug resistance in breast cancer by manipulating GCS and glycolipids, and as such, it represents a major shift in the research paradigm for drug resistance. This is also the first study to propose an approach that might have prophylactic as well as therapeutic value.

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INTRODUCTION

The development of resistance to cytotoxic drugs represents a formidable barrier to the successful treatment of cancer. Although some cancers such as melanoma can be intrinsically resistant, many cancers often acquire resistance through selection pressure in the face of adversity, e.g., chemotherapy. Unfortunately, almost half of all human breast cancers are resistant to doxorubicin (Adriamycin), a front-line chemotherapeutic agent. One of the most consistent biological alterations in drug resistance is overexpression of P-glycoprotein (P-gp).[1] This membrane-resident protein functions as a pump to reduce the intracellular concentration of anticancer drugs, chiefly natural product agents such as doxorubicin and paclitaxel (Taxol). Despite the strong association between P-gp and doxorubicin resistance in breast cancer,[2] the use of P-gp antagonists to reduce efflux of chemotherapy drugs has been disappointing. However, we believe that blocking P-gp may not be necessary; instead, we propose silencing the multidrug resistant (MDR1) phenotype by inhibition of glycolipid metabolism. The ceramide that is generated in doxorubicin-resistant breast cancer cells undergoes an abnormally high rate of glycosylation by glucosylceramide synthase (GCS), and there is a direct correlation between drug resistance, GCS, and the MDR1 phenotype.[3] The relationship between MDR1 and GCS has also been demonstrated by transfection of drug-resistant breast cancer cells with antisense GCS: levels of MDR1 mRNA and MDR1 protein (P-gp) decreased, while cell sensitivity to doxorubicin and paclitaxel increased by factors of 40 and 200, respectively; transfected cells also contained 10-fold more drug than nontransfected cells.[4] Chemical inhibition of GCS can also produce increased sensitivity to chemotherapy.[5] These data indicate that glycolipids exert a role in the expression of multidrug resistance. Because multidrug-resistant breast cancer cells can be chemosensitized by antisense GCS transfection and by chemical inhibition of GCS with agents such as PPMP, the glycolipid metabolic pathway catalyzed by GCS must be pivotal for expression of the MDR1 phenotype. Our study will determine whether blocking the glycosylation of ceramide can be therapeutic in drug-resistant cells and prophylactic in drug-sensitive cells. If blocking ceramide glycosylation limits expression of MDR1, than therapeutic advantages can be realized.

BODY

Task 1 of our project was to determine how inhibitors of GCS affect MDR1/P-gp expression and chemotherapy sensitivity in drug-resistant breast cancer cells. Our underlying hypothesis is that glycolipids, in particular glucosylceramide, upregulate MDR1 expression. Experiments were initiated using the highly-MDR1 overexpressing human breast cancer cell line, MCF-7-AdrR, and we investigated whether antisense GCS transfection, which will downregulate glycolipid production, impacted the expression levels of MDR1.

A reduction in GCS activity (by antisense transfection) would be expected to lower cellular glycolipid levels. In these experiments, we employed a stably transfected (antisense GCS) MCF-7-AdrR cell line, termed MCF-7-AdrR/asGCS. Isolation and derivatization of cellular gangliosides followed by quantitative analysis revealed that the ganglioside content was reduced nearly 4-fold in MCF-7-AdrR/asGCS cells compared to MCF-7-AdrR cells (Figure 1, left). The influence of downregulating GCS expression on MDR1 expression was dramatic (Figure 1, middle and right); asGCS-transfected cells were nearly devoid of MDR1 mRNA (RT-PCR, middle) and P-gp protein (Western blot, right).

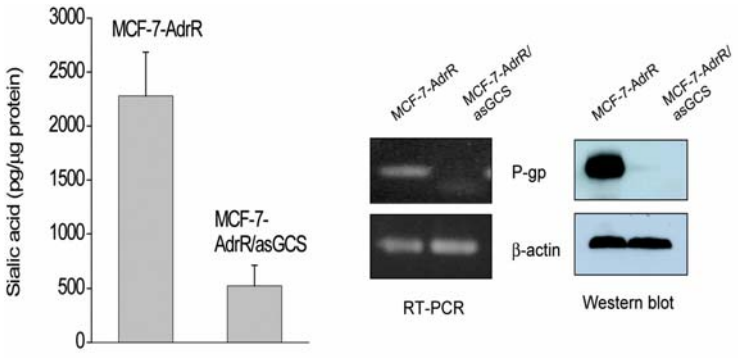

Figure 1. Antisense GCS transfection lowers cellular ganglioside levels and diminishes MDR1 expression. Left, Gangliosides were extracted and quantitated by sialic acid derivitization. Middle and Right, MDR1 expression by RT-PCR and by Western blot.

Using a commercial database, we obtained a working small interfering RNA (siRNA) to GCS. The use of inhibitory agents to retard GCS activity and depress GC (and ganglioside synthesis) has been helpful in assessing the influence of GCS on MDR1. The data in Figure 2 demonstrate that a specific inhibitor of GCS, in this case PPMP (Figure 2, left), and a nonspecific inhibitor of GCS, in this case tamoxifen (Figure 2, middle), and a gene knockdown technique (Figure 2, right) greatly deplete or eliminate MDR1 expression in multidrug-resistant human breast cancer cells (exposed to pharmacologically attainable concentrations of these agents). With tamoxifen, reduction in the expression of MDR1 was time-dependent. The employ of siRNA, to specifically knockdown GCS, demonstrated nearly complete depletion of both GCS and MDR1 mRNA, while β-actin control remained unaltered (Figure 2, right).

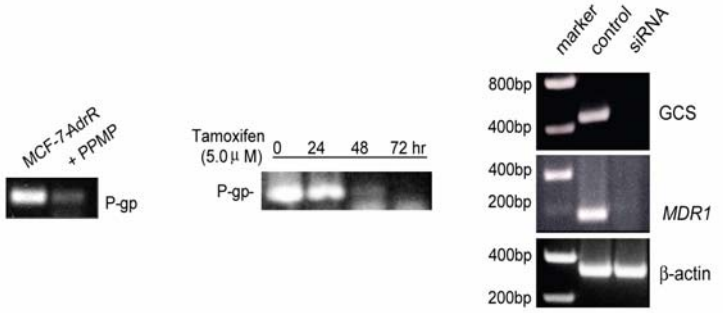

Figure 2. GCS blockers decrease expression of MDR1 in multidrug resistant MCF-7-AdrR cells. Left, MCF-7-AdrR cells were grown with 5.0 µM of PPMP for 48 hours. Middle, MCF-7-AdrR cells were grown with 5.0 µM of tamoxifen for times shown. Right, Cells were treated with GCS siRNA (100 nM) for 4 hr and continued in FBS-containing medium for 48 hr. Control, lipofectamine regents.

The D,L-*erythro* form of PPMP is inactive as a GCS inhibitor. We thus tested D,L-*erythro*-PPMP to determine whether this stereo isomer influenced cellular MDR1 expression. The results of realtime RT-PCR showed that MDR1 expression in MCF-7-AdrR cells treated with D,L-*threo*-PPMP and D,L-*erythro*-PPMP (48 hr) was reduced by 60% and 12%, respectively, compared to untreated control cultures. This demonstrates that downregulation of P-gp by PPMP is stereo-specific and thus selectively via inhibition of glycolipid metabolism.

Other key research accomplishments centered on the impact of GCS on drug uptake. The hypothesis behind these studies was that limiting GCS activity would heighten drug uptake (and increase drug sensitivity) because MDR1 would be downregulated under these circumstances. Using antisense GCS as a GCS-limiting technique, we demonstrated that cellular uptake of both vinblastine and paclitaxel was enhanced nearly 10-fold, compared to MCF-7-AdrR cells. Similarly, using D,L-*threo*-PPMP to limit GCS activity – in place of antisense GCS – and we evaluated both chemotherapy uptake and cellular viability. These experiments would tell us whether "chemical" lowering (use of PPMP) of MDR1 expression affects breast cancer cell response to chemotherapy. Treatment of MCF-7-AdrR cells with D,L-*threo*-PPMP improved vinblastine uptake by 3-fold and improved vinblastine cytotoxicity compared to PPMP naïve controls. For example, 100 nM vinblastine in the presence of PPMP affected a 60% cell kill compared to no cell kill in the absence of PPMP.

RU486 (Mifepristone) is now FDA-approved. We were the first to discover that this anti-progestine inhibited production of glycolipids,[6] and one goal in the present study was to determine whether RU486 would enhance chemotherapy sensitivity. Our work shows that RU486 is not cytotoxic (5 and 10 μM) in MCF-7-AdrR cells; however, when mixed with doxorubicin, RU486 enhanced cytotoxicity in a synergistic manner. For example, viability in MCF-7-AdrR cells exposed to 1.0 μM doxorubicin was 75%, and with 5.0 μM RU486 viability was 100%. When MCF-7-AdrR cells were exposed to a mixed regimen, viability fell to 10%. Glucosylceramide production was inhibited by 50% in cells exposed to 5 μM RU486.

In Task 2, we sought to determine whether GCS inhibitors would forestall acquired expression of MDR1, using MCF-7 cells as a model (these are chemotherapy-naïve cells). We have determined the influence of doxorubicin exposure on the induction of MDR1. MCF-7 cells were treated with 0.5 microM doxorubicin for increasing times and MDR1 gene expression was analyzed by realtime RT-PCR. MDR1 mRNA levels remained relatively baseline (compared to untreated cells) until 48 hr at which time gene expression increased 6-fold (over doxorubicin-free cells). At 72 hr, MDR1 expression increased to 8.5-fold over control. Therefore, the induction of MDR1 in breast cancer cells is measurable within a period of 48 hr. Pre-treatment of MCF-7 cells with 10 microM PPMP for 2 hr before doxorubicin addition, diminished the increase in MDR1 expression by 50%. These results show that diminishing the activity of GCS (limiting ceramide glycosylation) retards doxorubicin-induced expression of MDR1. It is noteworthy that even though PPMP retarded doxorubicin-induced expression of MDR1 by 50%, no differences in cell sensitivity to anticancer agents were demonstrated. This is likely due to the low number of gene copies (8.5-fold increase reduced to 4.5-fold) not having a significant influence on actual expression of P-gp.

Although not a specific part of our Statement of Work but related to our hypothesis that glycolipids upregulate MDR1 expression, we demonstrated that exposure of human breast cancer cells to the simple glycolipid, glucosylceramide, upregulated MDR1 expression. These experiments were conducted with several human breast cancer cell lines, including MCF-7, MDA-MB-231, MDA-MB-435, and T47D. These results are captioned in an abstract presented at the 18th EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics, 7-10 November 2006, and listed here under "Reportable Outcomes". Briefly, with MDA-MB-231 cells as the model, exposing cells to either C8-ceramide or C8-glucosylceramide (cell-permeable analogs of natural molecular counterparts) enhanced expression of the MDR1 phenotype in these wild-type MDR1-naïve cells. Experiments on C8-ceramide metabolism in these cells showed conversion of the supplement to C8-glucosylceramide. Interestingly, growth of MDA-MB-231 cells with c8-ceramide (5 microgram/ml medium) for extended passages enhanced MDR1 mRNA levels and P-gp levels and decreased cellular sensitivity to doxorubicin and paclitaxel. This is demonstrated in Figure 3, where it is shown by Western blot that MDA-MB-231 cells are devoid of P-gp but growth of cells for 12 and 22 passages enhances the expression of this ABC transporter. A manuscript has been submitted to ***Biochim Biophys Acta, Molecular and Cell Biology of Lipids***.

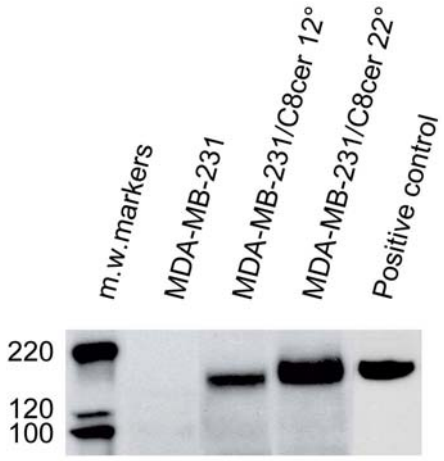

Figure 3. Growth of MDA-MB-231 cells with C8-ceramide increases P-gp expression. Cells were grown for 3 passages in 2.5 microgram/ml medium C8-ceramide before increase to 5 microgram/ml. P-gp is demonstrated by Western blot using C219 monoclonal antibody. Positive control is KB-Ch-8-5 cells (colchicine-resistant).

KEY RESEARCH ACCOMPLISHMENTS

asGCS-transfection of multidrug-resistant MCF-7-AdrR cells lowers MDR1 and P-gp expression

Knocking down GCS in MCF-7-AdrR cells with GCS siRNA eliminates GCS expression, as well it should, but also eliminates MDR1 expression.

Specific chemical inhibition of GCS by PPMP downregulates P-gp expression in MCF-7-AdrR cells.

Nonspecific inhibition of GCS by tamoxifen downregulates P-gp expression in MCF-7-AdrR cells.

Inhibiting GCS enhances chemotherapy uptake and sensitivity in MCF-7-AdrR cells. RU486 is also effective.

The addition of PPMP to MCF-7 wild-type breast cancer cells forestalls doxorubicin-induced upregulation of MDR1.

Exposure of wild-type human breast cancer cells to either ceramide or glucosylceramide upregulates expression of the multidrug-resistant phenotype.

REPORTABLE OUTCOMES

Gouaze-Andersson V, Yu J, Kreitenberg A, Giuliano AE, Cabot M. Sphingolipids enhance expression of the multidrug resistance phenotype in human breast cancer cells (abstract). Presented at the 18th EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics, 7-10 November 2006, Prague, Czech Republic.

CONCLUSIONS

This is the first study to attack drug-resistant human breast cancer by manipulating GCS and glycolipids, and as such, it represents a major shift in the research paradigm for drug resistance. This is also the first study to propose an approach that might have prophylactic as well as therapeutic value. The experiments showing that MDR1 expression can be lowered by introducing inhibitors of glycolipid synthesis are encouraging, and because RU486 and tamoxifen are effective (and already approved for use), translation of these findings to the clinic are within reach. Additionally, PPMP is approved for use in treatment of Gaucher's disease, making this agent also attractive for treatment of drug-resistant breast cancer. Our experiments showing that the addition of PPMP to wild-type breast cancer cells depresses doxorubicin-induced MDR1 expression are also encouraging. Finally, this is the first report showing that exposure of breast cancer cells to lipids, in this case sphingolipids with ceramide backbones, promotes the expression of the multidrug-resistant phenotype.

Overall, we conclude that this approach may be a way to limit the expression of drug resistance in breast cancer patients receiving chemotherapy. We conclude that research of this type could be of value in the treatment setting, and we believe that this avenue should be pursued further, especially as regards the possible utility of GCS inhibitors to forestall the onset of drug resistance caused by MDR1.

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APPENDICES

None.